IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Nobuaki TAKAHASHI *et al.*

Title: **ANTI-CD40 ANTIBODY MUTANTS**

Appl. No.: 10/584,345

Examiner: Phillip GAMBEL

Art Unit: 1644

Confirmation Number: 3671

DECLARATION OF DR. NOBUAKI TAKAHASHI UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Dr. Nobuaki Takahashi, hereby declare and state as follows:

1. I currently hold the position of Senior Scientist in the Antibody Research Laboratories Research Division of KYOWA HAKKO KIRIN CO., LTD. ("KHK"), Japan, the assignee of the captioned application.

2. I have been employed by KHK since 1995, when I obtained my Ph.D. from Osaka University. I worked at Gemini Science, Inc., a subsidiary of KHK in the U.S., from 1998 to 2000. In that position I generated and evaluated various monoclonal antibodies. Since that time I have worked on the research and development of monoclonal antibodies.

3. I am a co-inventor of the captioned application, in relation to which I have submitted a previous declaration. It is expected that I would be remunerated when KHK gains income from commercializing a product or technology that is related to the invention of the application.

4. I understand that the application is directed to an antagonistic CD40 antibody designated "4D11G4PE;" compositions that comprise the 4D11G4PE antibody; and a method of treating or preventing transplant rejection by administration of the 4D11G4PE antibody.

5. The 4D11G4PE antibody, a mutant of the 4D11 antibody, is an anti-CD40 antagonistic antibody. Relative to the 4D11 antibody, the 4D11G4PE antibody contains two amino acid substitutions at positions S228P and L235E, as indicated by the EU index of Kabat *et al.*, a standard antibody notation system.

6. While employed with KHK, I was involved in experiments to determine whether any agonistic activity could be observed upon *in vivo* administration of the 4D11G4PE antibody. In particular, I was involved in experiments to determine what, if any, agonistic activity arose from administration of 100 mg/kg of the antibody in cynomolgus monkeys. These experiments and their results are summarized below.

7. Phosphate buffered saline (PBS) alone (tests #1 and #2) or combined with 100 mg/kg of 4D11G4PE (tests #3-#5) was administered intravenously to male cynomolgus monkeys for four weeks, once weekly. Blood was drawn from the femoral vein of the monkeys at indicated time points and was analyzed for IL-12 or IFNγ by ELISA.

8. The results are summarized in the following tables. Further details of the experimental methodology and results are set out in an attachment to this declaration.

Table 1. ***Serum IL-12 concentrations upon administration of 4D11G4PE***

Test no.	Before administration	24 h after 1st administration	24 h after 4th administration	7 d after 4th administration
#1	BLOQ	BLOQ	BLOQ	43.1
#2	41.9	13.7	19.4	29.0
#3	BLOQ	BLOQ	15.7	10.4
#4	128.7	82.3	23.6	93.6
#5	58.1	25.3	BLOQ	BLOQ
BLOQ (below the limit of quantification)				

Table 2. ***Serum IFNγ concentrations upon administration of 4D11G4PE***

Test no.	Before administration	24 h after 1st administration	24 h after 4th administration	7 d after 4th administration
#1	47.7	BLOQ	BLOQ	27.1
#2	BLOQ	BLOQ	BLOQ	42.1
#3	BLOQ	BLOQ	BLOQ	BLOQ
#4	4.3	72.7	18.2	34.3
#5	85.4	102.7	64.3	69.3
BLOQ (below the limit of quantification)				

9. These data demonstrate that the 4D11G4PE antibody shows not simply a reduction in but rather a negation of agonistic activity at a concentration of 100 mg/kg. The nominal variations or apparent "increases" in value are biologically insignificant.

10. I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date _____ By _____

Dr. Nobuaki Takahashi

Cytokine Measurement in Serum Samples Collected in "4-Week Intermittent Intravenous Dose Toxicity Study of 4D11G4PE in Cynomolgus Monkeys"

1 SUMMARY

PBS as vehicle control and 100 mg/kg of 4D11G4PE were administered intravenously once weekly for 4 weeks to two and three male cynomolgus monkeys, respectively. Even though the 4D11G4PE serum concentration level exceeded more than 2 mg/mL just after 4D11G4PE administration, no test article-related changes were noted in serum IL-12 and IFN-g (GAMMA) level.

2 INTRODUCTION

Ligation of CD40 induces production of some inflammatory cytokines, such as IL-12 and IFN-g. In this study, it was assessed whether high dose of 4D11G4PE caused agonistic effects.

3 MATERIALS AND METHODS

3.1 4-Week Intermittent Intravenous Dose Toxicity Study of 4D11G4PE in Cynomolgus Monkeys

Briefly, PBS as vehicle control and 100 mg/kg of 4D11G4PE were administered intravenously once weekly for 4 weeks to two and three male cynomolgus monkeys, respectively, in order to investigate its toxicity.

1 control group and 1 test article groups

Group	Test and control articles	Dose Level (mg/kg)	Dose Volume (mL/kg)	Concentration (mg/mL)	Number of Animal (Animal Number)
1	vehicle	-	10.3	-	2 (1, 2)
2	4D11G4PE	100	10.3	9.74	3 (3～5)

Blood was drawn from the femoral vein with a syringe before the first administration of 4D11G4PE, 24 hours after the first administration, 24 hours after the forth administration, and 7 days after fourth administration for serum cytokine measurement. The blood was left at room temperature for 20 to 60 minutes, and serum was obtained by centrifugation (room temperature, 1710×g, 3000 rpm, 15 minutes). The serum was stored in a deep freezer until cytokine measurement.

3.2 Measurement of serum 4D11G4PE concentration

Toxicological kinetics were measured as follows.

1) Recombinant soluble CD40 as capture antigen was diluted with PBS to make a 1 μg/mL solution.

2) Prepared soluble CD40 solution (coating solution, 100 μL/well) was added to wells of an ELISA plate and the plate was kept at 4 degrees for 16 to 24 hours.

3) The coating solution was discarded, and 1%BSA/PBS (300 μL/well, blocking buffer) was added to the wells.

4) The plate was placed at room temperature for 2 hours.

5) The blocking buffer was discarded, and 10-fold diluted calibration standard solutions and TK samples (100 μL/well) were added to the wells.

6) The plate was placed at room temperature for 2 hours.

7) All sample solution were discarded, and 0.1%Tween20/0.5M-$NaCl_2$/PBS (300 μL/well, washing buffer) was added to the wells, and then the washing buffer was discarded. This washing procedure was repeated 5 times.

8) Secondary antibody (Anti-Human Kappa Light Chain Goat IgG, HRP labeled) was diluted 5000 times.

9) Prepared secondary antibody solution (100 μL/well) was added to the wells .

10) The plate was placed on at room temperature for 1 hour.

11) The secondary antibody solution (300 μL/well) was discarded, and washing buffer was added to the wells, and then the washing buffer was discarded. This washing procedure was repeated 5 times .

12) Peroxidase substrate buffer (100 μL/well) was added to the wells and placed at room temperature for 20 minutes.

13) Stop buffer (100 μL/well) was added to the well.

14) Absorbance at 450 nm was measured.

3.3 Measurement of serum cytokine concentration

Serum samples were thawed slowly in warm water while gently shaking just before cytokine measurement. Cytokine measurement was conducted nsing Monkey IFN-g ELISA kit (Biosource, Cat# KPC4022 and Monkey IL-12 ELISA kit (Biosource, Cat# KPC0122) according to manufacturer's protocol.

4 RESULTS

Exposure increased gradually by repeated dosing(Fig 1). The mean Cmax of the first and the last administration were 2.82 and 3.69 mg/mL, respectively. The trough level of just before the second and the last administration 0.613 and 1.16 mg/mL, respectively.

No test article-related changes were noted in IL-12 and IFN-g level in any group even in repeated high dose of 4D11G4PE (Fig 2 and Table 1 for IL-12, Fig 3 and Table 2 for IFN-g).

5 DISCUSSION

4D11G4PE seems to be a pure antagonistic antibody, and did not behave as agonist even when it was repeatedly administered 4 times weekly at the dose of 100 mg/kg.

6 FIGURES and TABLES

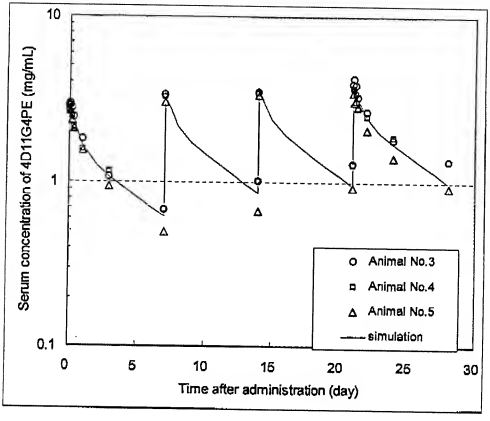

Figure. 1 Serum concentration of 4D11G4PE

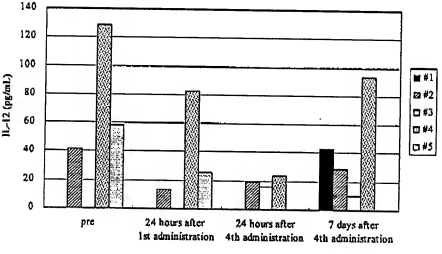

Figure.2 Serum IL-12 concentration before and after administration of 4D11G4PE

	pre	24 hours after 1st administration	24 hours after 4th administration	7 days after 4th administration
#1	BLOQ	BLOQ	BLOQ	43.1
#2	41.9	13.7	19.4	29.0
#3	BLOQ	BLOQ	15.7	10.4
#4	128.7	82.3	23.6	93.6
#5	58.1	25.3	BLOQ	BLOQ

BLOQ (below the limit of quantification)

Table 1 Serum IL-12 concentration before and after administration of 4D11G4PE

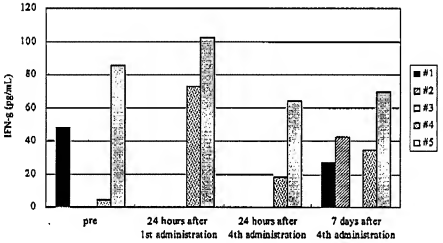

Figure.3 Serum IFN-g concentration before and after administration of 4D11G4PE

	pre	24 hours after 1st administration	24 hours after 4th administration	7 days after 4th administration
#1	47.7	BLOQ	BLOQ	27.1
#2	BLOQ	BLOQ	BLOQ	42.1
#3	BLOQ	BLOQ	BLOQ	BLOQ
#4	4.3	72.7	18.2	34.3
#5	85.4	102.7	64.3	69.3

BLOQ (below the limit of quantification)

Table 2 Serum IFN-g concentration before and after administration of 4D11G4PE